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(54) **Fusion proteins containing N-terminal fragments of human serum albumin.**

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**EP-A- 0 308 381
EP-A- 0 322 094**

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Description

The present invention relates to fusion polypeptides where two individual polypeptides or parts thereof are fused to form a single amino acid chain. Such fusion may arise from the expression of a single continuous coding sequence formed by recombinant DNA techniques.

Fusion polypeptides are known, for example those where a polypeptide which is the ultimately desired product of the process is expressed with an N-terminal "leader sequence" which encourages or allows secretion of the polypeptide from the cell. An example is disclosed in EP-A-116 201 (Chiron).

Human serum albumin (HSA) is a known protein found in the blood. EP-A-147 198 (Delta Biotechnology) discloses its expression in a transformed host, in this case yeast. Our earlier application EP-A-322 094 discloses N-terminal fragments of HSA, namely those consisting of residues 1-n where n is 369 to 419, which have therapeutic utility. The application also mentions the possibility of fusing the C-terminal residue of such molecules to other, unnamed, polypeptides.

One aspect of the present invention provides a fusion polypeptide comprising, as at least part of the N-terminal portion thereof, an N-terminal portion of HSA or a variant thereof and, as at least part of the C-terminal portion thereof, another polypeptide except that, when the said N-terminal portion of HSA is the 1-n portion where n is 369 to 419 or a variant thereof then the said polypeptide is (a) the 585 to 1578 portion of human fibronectin or a variant thereof, (b) the 1 to 368 portion of CD4 or a variant thereof, (c) platelet derived growth factor, or a variant thereof, (d) transforming growth factor, or a variant thereof, (e) the 1-261 portion of mature human plasma fibronectin or a variant thereof, (f) the 278-578 portion of mature human plasma fibronectin or a variant thereof, (g) the 1-272 portion of mature human von Willebrand's Factor or a variant thereof, or (h) alpha-1-antitrypsin or a variant thereof.

The N-terminal portion of HSA is preferably the said 1-n portion, the 1-177 portion (up to and including the cysteine), the 1-200 portion (up to but excluding the cysteine) or a portion intermediate 1-177 and 1-200.

The term "human serum albumin" (HSA) is intended to include (but not necessarily to be restricted to) known or yet-to-be-discovered polymorphic forms of HSA. For example, albumin Naskapi has Lys-372 in place of Glu-372 and pro-albumin Christchurch has an altered pro-sequence. The term "variants" is intended to include (but not necessarily to be restricted to) minor artificial variations in sequence (such as molecules lacking one or a few residues, having conservative substitutions or minor insertions of residues, or having minor variations of amino acid structure). Thus polypeptides which have 80%, preferably 85%, 90%, 95% or 99%, homology with HSA are deemed to be "variants". It is also preferred for such variants to be physiologically equivalent to HSA; that is to say, variants preferably share at least one pharmacological utility with HSA. Furthermore, any putative variant which is to be used pharmacologically should be non-immunogenic in the animal (especially human) being treated.

Conservative substitutions are those where one or more amino acids are substituted for others having similar properties such that one skilled in the art of polypeptide chemistry would expect at least the secondary structure, and preferably the tertiary structure, of the polypeptide to be substantially unchanged. For example, typical such substitutions include asparagine for glutamine, serine for asparagine and arginine for lysine. Variants may alternatively, or as well, lack up to ten (preferably only one or two) intermediate amino acid residues (ie not at the termini of the said N-terminal portion of HSA) in comparison with the corresponding portion of natural HSA; preferably any such omissions occur in the 100 to 369 portion of the molecule (relative to mature HSA itself) (if present). Similarly, up to ten, but preferably only one or two, amino acids may be added, again in the 100 to 369 portion for preference (if present). The term "physiologically functional equivalents" also encompasses larger molecules comprising the said sequence plus a further sequence at the N-terminal (for example, pro-HSA, pre-pro-HSA and met-HSA).

Clearly, the said "another polypeptide" in the fusion compounds of the invention cannot be the remaining portion of HSA, since otherwise the whole polypeptide would be HSA, which would not then be a "fusion polypeptide".

Even when the HSA-like portion is not the said 1-n portion of HSA, it is preferred for the non-HSA portion to be one of the said (a) to (h) entities.

The 1 to 368 portion of CD4 represents the first four disulphide-linked immunoglobulin-like domains of the human T lymphocyte CD4 protein, the gene for and amino acid sequence of which are disclosed in D. Smith *et al* (1987) *Science* **328**, 1704-1707. It is used to combat HIV infections.

The sequence of human platelet-derived growth factor (PDGF) is described in Collins *et al* (1985) *Nature* **316**, 748-750. Similarly, the sequence of transforming growth factors β (TGF- β) is described in Derynck *et al* (1985) *Nature* **316**, 701-705. These growth factors are useful for wound-healing.

A cDNA sequence for the 1-261 portion of Fn was disclosed in EP-A-207 751 (obtained from plasmid pFH6 with endonuclease *PvuII*). This portion binds fibrin and can be used to direct fused compounds to blood clots.

A cDNA sequence for the 278-578 portion of Fn, which contains a collagen-binding domain, was disclosed by R.J. Owens and F.E. Baralle in 1986 E.M.B.O.J. 5, 2825-2830. This portion will bind to platelets.

The 1-272 portion of von Willebrand's Factor binds and stabilises factor VIII. The sequence is given in Bontham *et al*, Nucl. Acids Res. 14, 7125-7127.

5 Variants of alpha-1-antitrypsin include those disclosed by Rosenberg *et al* (1984) Nature 312, 77-80. In particular, the present invention includes the Pittsburgh variant (Met³⁵⁸ is mutated to Arg) and the variant where Pro³⁵⁷ and Met³⁵⁸ are mutated to alanine and arginine respectively. These compounds are useful in the treatment of septic shock and lung disorders.

10 Variants of the non-HSA portion of the polypeptides of the invention include variations as discussed above in relation to the HSA portion, including those with conservative amino acid substitutions, and also homologues from other species.

The fusion polypeptides of the invention may have N-terminal amino acids which extend beyond the portion corresponding to the N-terminal portion of HSA. For example, if the HSA-like portion corresponds to an N-terminal portion of mature HSA, then pre-, pro-, or pre-pro sequences may be added thereto, for example 15 the yeast alpha-factor leader sequence. The fused leader portions of WO 90/01063 may be used. The polypeptide which is fused to the HSA portion may be a naturally-occurring polypeptide, a fragment thereof or a novel polypeptide, including a fusion polypeptide. For example, in Example 3 below, a fragment of fibronectin is fused to the HSA portion via a 4 amino acid linker.

It has been found that the amino terminal portion of the HSA molecule is so structured as to favour particularly efficient translocation and export of the fusion compounds of the invention in eukaryotic cells.

A second aspect of the invention provides a transformed host having a nucleotide sequence so arranged as to express a fusion polypeptide as described above. By "so arranged", we mean, for example, that the nucleotide sequence is in correct reading frame with an appropriate RNA polymerase binding site and translation start sequence and is under the control of a suitable promoter. The promoter may be homologous with or heterologous to the host. Downstream (3') regulatory sequences may be included if desired, as is known. The 25 host is preferably yeast (for example *Saccharomyces* spp., e.g. *S. cerevisiae*; *Kluyveromyces* spp., e.g. *K. lactis*; *Pichia* spp.; or *Schizosaccharomyces* spp., e.g. *S. pombe*) but may be any other suitable host such as *E. coli*, *B. subtilis*, *Aspergillus* spp., mammalian cells, plant cells or insect cells.

A third aspect of the invention provides a process for preparing a fusion polypeptide according to the first 30 aspect of the invention by cultivation of a transformed host according to the second aspect of the invention, followed by separation of the fusion polypeptide in a useful form.

A fourth aspect of the invention provides therapeutic methods of treatment of the human or other animal body comprising administration of such a fusion polypeptide.

35 In the methods of the invention we are particularly concerned to improve the efficiency of secretion of useful therapeutic human proteins from yeast and have conceived the idea of fusing to amino-terminal portions of HSA those proteins which may ordinarily be only inefficiently secreted. One such protein is a potentially valuable wound-healing polypeptide representing amino acids 585 to 1578 of human fibronectin (referred to herein as Fn 585-1578). As we have described in a separate application (filed simultaneously herewith) this molecule contains cell spreading, chemotactic and chemokinetic activities useful in healing wounds. The fusion 40 polypeptides of the present invention wherein the C-terminal portion is Fn 585-1578 can be used for wound healing applications as biosynthesised, especially where the hybrid human protein will be topically applied. However, the portion representing amino acids 585 to 1578 of human fibronectin can if desired be recovered from the fusion protein by preceding the first amino acid of the fibronectin portion by amino acids comprising a factor X cleavage site. After isolation of the fusion protein from culture supernatant, the desired molecule is 45 released by factor X cleavage and purified by suitable chromatography (e.g. ion-exchange chromatography). Other sites providing for enzymatic or chemical cleavage can be provided, either by appropriate juxtaposition of the N-terminal and C-terminal portions or by the insertion therebetween of an appropriate linker.

At least some of the fusion polypeptides of the invention, especially those including the said CD4 and vWF fragments, PDGF and α_1 AT, also have an increased half-life in the blood and therefore have advantages and 50 therapeutic utilities themselves, namely the therapeutic utility of the non-HSA portion of the molecule. In the case of α_1 AT and others, the compound will normally be administered as a one-off dose or only a few doses over a short period, rather than over a long period, and therefore the compounds are less likely to cause an immune response.

55 EXAMPLES : SUMMARY

Standard recombinant DNA procedures were as described by Maniatis *et al* (1982 and recent 2nd edition) unless otherwise stated. Construction and analysis of phage M13 recombinant clones was as described by

Messing (1983) and Sanger et al (1977).

DNA sequences encoding portions of human serum albumin used in the construction of the following molecules are derived from the plasmids mHOB12 and pDBD2 (EP-A-322 094, Delta Biotechnology Ltd, relevant portions of which are reproduced below) or by synthesis of oligonucleotides equivalent to parts of this sequence. DNA sequences encoding portions of human fibronectin are derived from the plasmid pFHDEL1, or by synthesis of oligonucleotides equivalent to parts of this sequence. Plasmid pFHDEL1, which contains the complete human cDNA encoding plasma fibronectin, was obtained by ligation of DNA derived from plasmids pFH6, 16, 54, 154 and 1 (EP-A-207 751; Delta Biotechnology Ltd).

This DNA represents an mRNA variant which does not contain the 'ED' sequence and had an 89-amino acid variant of the III-CS region (R.J. Owens, A.R. Kornblihtt and F.E. Baralle (1986) Oxford Surveys on Eukaryotic Genes 3 141-160). The map of this vector is disclosed in Fig. 11 and the protein sequence of the mature polypeptide produced by expression of this cDNA is shown in Fig. 5.

Oligonucleotides were synthesised on an Applied Biosystems 380B oligonucleotide synthesiser according to the manufacturer's recommendations (Applied Biosystems, Warrington, Cheshire, UK).

An expression vector was constructed in which DNA encoding the HSA secretion signal and mature HSA up to and including the 387th amino acid, leucine, fused in frame to DNA encoding a segment of human fibronectin representing amino acids 585 to 1578 inclusive, was placed downstream of the hybrid promoter of EP-A-258 067 (Delta Biotechnology), which is a highly efficient galactose-inducible promoter functional in *Saccharomyces cerevisiae*. The codon for the 1578th amino acid of human fibronectin was directly followed by a stop codon (TAA) and then the *S. cerevisiae* phosphoglycerate kinase (PGK) gene transcription terminator. This vector was then introduced into *S. cerevisiae* by transformation, wherein it directed the expression and secretion from the cells of a hybrid molecule representing the N-terminal 387 amino acids of HSA C-terminally fused to amino acids 585 to 1578 of human fibronectin.

In a second example a similar vector is constructed so as to enable secretion by *S. cerevisiae* of a hybrid molecule representing the N-terminal 195 amino acids of HSA C-terminally fused to amino acids 585 to 1578 of human fibronectin.

Aspects of the present invention will now be described by way of example and with reference to the accompanying drawings, in which:

Figure 1 (on two sheets) depicts the amino acid sequence currently thought to be the most representative of natural HSA, with (boxed) the alternative C-termini of HSA(1-n);

Figure 2 (on two sheets) depicts the DNA sequence coding for mature HSA, wherein the sequence included in Linker 3 is underlined;

Figure 3 illustrates, diagrammatically, the construction of mHOB16;

Figure 4 illustrates, diagrammatically, the construction of pHOB31;

Figure 5 (on 6 sheets) illustrates the mature protein sequence encoded by the Fn plasmid pFHDEL1;

Figure 6 illustrates Linker 5, showing the eight constituent oligonucleotides;

Figure 7 shows schematically the construction of plasmid pDBDF2;

Figure 8 shows schematically the construction of plasmid pDBDF5;

Figure 9 shows schematically the construction of plasmid pDBDF9;

Figure 10 shows schematically the construction of plasmid DBDF12, using plasmid pFHDEL1; and

Figure 11 shows a map of plasmid pFHDEL1.

EXAMPLE 1 : HSA 1-387 FUSED TO Fn 585-1578

The following is an account of a preparation of plasmids comprising sequences encoding a portion of HSA, as is disclosed in EP-A-322 094.

The human serum albumin coding sequence used in the construction of the following molecules is derived from the plasmid M13mp19.7 (EP-A-201 239, Delta Biotechnology Ltd.) or by synthesis of oligonucleotides equivalent to parts of this sequence. Oligonucleotides were synthesised using phosphoramidite chemistry on an Applied Biosystems 380B oligonucleotide synthesizer according to the manufacturer's recommendations (AB Inc., Warrington, Cheshire, England).

An oligonucleotide was synthesised (Linker A) which represented a part of the known HSA coding sequence (Figure 2) from the *Pst*I site (1235-1240, Figure 2) to the codon for valine 381 wherein that codon was changed from GTG to GTC:

Linker 1

		D	P	H	E	C	Y		
5	5'	GAT	CCT	CAT	GAA	TGC	TAT		
	3' ACGT	CTA	GGA	GTA	CTT	ACG	ATA		
		1247							
10									
		A	K	V	F	D	E	F	K
15	GCC	AAA	GTG	TTC	GAT	GAA	TTT	AAA	
	CGG	TTT	CAC	AAG	CTA	CTT	AAA	TTT	
		1267							
20	P	L	V						
	CTT	GTC	3'						
25	GGA	CAG	5'						

Linker 1 was ligated into the vector M13mp19 (Norrander *et al.*, 1983) which had been digested with PstI and HincII and the ligation mixture was used to transfect E.coli strain XL1-Blue (Stratagene Cloning Systems, San Diego, CA). Recombinant clones were identified by their failure to evolve a blue colour on medium containing the chromogenic indicator X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactoside) in the presence of IPTG (isopropylthio- β -galactoside). DNA sequence analysis of template DNA prepared from bacteriophage particles of recombinant clones identified a molecule with the required DNA sequence, designated mHOB12 (Figure 3).

M13mp19.7 consists of the coding region of mature HSA in M13mp19 (Norrander *et al.*, 1983) such that the codon for the first amino acid of HSA, GAT, overlaps a unique XhoI site thus:

		Asp	Ala
	5'	C T C G A G A T G C A	3'
40	3'	G A G C T C T A C G T	5'
		<u>Xho</u> I	

(EP-A-210 239). M13mp19.7 was digested with XhoI and made flush-ended by S1-nuclease treatment and was then ligated with the following oligonucleotide (Linker 2):

Linker 2

50	5'	T C T T T T A T C C A A G C T T G G A T A A A A G A	3'
	3'	A G A A A A T A G G T T C G A A C C T A T T T T C T	5'
55		<u>Hind</u> III	

The ligation mix was then used to transfect E.coli XL1-Blue and template DNA was prepared from several

plaques and then analysed by DNA sequencing to identify a clone, pDBD1 (Figure 4), with the correct sequence.

A 1.1 kb HindIII to PstI fragment representing the 5' end of the HSA coding region and one half of the inserted oligonucleotide linker was isolated from pDBD1 by agarose gel electrophoresis. This fragment was then ligated with double stranded mHOB12 previously digested with HindIII and PstI and the ligation mix was then used to transfect E.coli XL1-Blue. Single stranded template DNA was prepared from mature bacteriophage particles of several plaques. The DNA was made double stranded in vitro by extension from annealed sequencing primer with the Klenow fragment of DNA polymerase I in the presence of deoxynucleoside triphosphates. Restriction enzyme analysis of this DNA permitted the identification of a clone with the correct configuration, mHOB15 (Figure 4).

The following oligonucleotide (Linker 3) represents from the codon for the 382nd amino acid of mature HSA (glutamate, GAA) to the codon for lysine 389 which is followed by a stop codon (TAA) and a HindIII site and then a BamHI cohesive end:

Linker 3

	E	E	P	Q	N	L	I	K	J		
5'	GAA	GAG	CCT	CAG	AAT	TTA	ATC	AAA	TAA	GCTTG	3'
3'	CTT	CTC	GGA	GTC	TTA	AAT	TAG	TTT	ATT	CGAACCTAG	5'

This was ligated into double stranded mHOB15, previously digested with HincII and BamHI. After ligation, the DNA was digested with HincII to destroy all non-recombinant molecules and then used to transfect E.coli XL1-Blue. Single stranded DNA was prepared from bacteriophage particles of a number of clones and subjected to DNA sequence analysis. One clone having the correct DNA sequence was designated mHOB16 (Figure 4).

A molecule in which the mature HSA coding region was fused to the HSA secretion signal was created by insertion of Linker 4 into BamHI and XhoI digested M13mp19.7 to form pDBD2 (Figure 4).

Linker 4

		M		K		W		V		S		F
5'	GATCC	ATG		AAG		TGG		GTA		AGC		TTT
		G	TAC		TTC		ACC		CAT		TCG	AAA
	I		S		L		L		F		L	
	ATT		TCC		CTT		CTT		TTT		CTC	
	TAA		AGG		GAA		GAA		AAA		GAG	
											AAA	TCG

S A Y S R G V F
 TCG GCT TAT TCC AGG GGT GTG TTT
 5 AGC CGA ATA AGG TCC CCA CAC AAA

 10 R R
 CG 3'
 GCAGCT 5'

15 In this linker the codon for the fourth amino acid after the initial methionine, ACC for threonine in the HSA pre-pro leader sequence (Lawn *et al.*, 1981), has been changed to AGC for serine to create a HindIII site.

A sequence of synthetic DNA representing a part of the known HSA coding sequence (Lawn *et al.*, 1981) (amino acids 382 to 387, Fig. 2), fused to part of the known fibronectin coding sequence (Kornblihtt *et al.*, 1985) (amino acids 585 to 640, Fig. 2), was prepared by synthesising six oligonucleotides (Linker 5, Fig. 6). The oligonucleotides 2, 3, 4, 6, 7 and 8 were phosphorylated using T4 polynucleotide kinase and then the oligonucleotides were annealed under standard conditions in pairs, i.e. 1+8, 2+7, 3+6 and 4+5. The annealed oligonucleotides were then mixed together and ligated with mHOB12 which had previously been digested with the restriction enzymes HincII and EcoRI. The ligation mixture was then used to transfect E.coli XL1-Blue (Stratagene Cloning Systems, San Diego, CA). Single stranded template DNA was then prepared from mature bacteriophage particles derived from several independent plaques and then was analysed by DNA sequencing. A clone in which a linker of the expected sequence had been correctly inserted into the vector was designated pDBDF1 (Fig. 7). This plasmid was then digested with PstI and EcoRI and the approx. 0.24kb fragment was purified and then ligated with the 1.29kb BamHI-PstI fragment of pDBD2 (Fig. 7) and BamHI + EcoRI digested pUC19 (Yanisch-Perron, *et al.*, 1985) to form pDBDF2 (Fig. 7).

A plasmid containing a DNA sequence encoding full length human fibronectin, pFHDEL1, was digested with EcoRI and XhoI and a 0.77kb EcoRI-xhoI fragment (Fig. 8) was isolated and then ligated with EcoRI and sall digested M13 mp18 (Norrandar *et al.*, 1983) to form pDBDF3 (Fig. 8).

The following oligonucleotide linker (Linker 6) was synthesised, representing from the PstI site at 4784-4791 of the fibronectin sequence of EP-A-207 751 to the codon for tyrosine 1578 (Fig. 5) which is followed by a stop codon (TAA), a HindIII site and then a BamHI cohesive end:

Linker 6

40 G P D Q T E M T I E G L
 GGT CCA GAT CAA ACA GAA ATG ACT ATT GAA GGC TTG
 45 A CGT CCA GGT CTA GTT TGT CTT TAC TGA TAA CTT CCG AAC

 Q P T V E Y Stop
 50 CAG CCC ACA GTG GAG TAT TAA GCTTG
 GTC GGG TGT CAC CTC ATA ATT CGAACCTAG

55 This linker was then ligated with PstI and HindIII digested pDBDF3 to form pDBDF4 (Fig. 8). The following DNA fragments were then ligated together with BglII digested pKV50 (EP-A-258 067) as shown in Fig. 8: 0.68kb EcoRI-BamHI fragment of pDBDF4, 1.5kb BamHI-StuI fragment of pDBDF2 and the 2.2kb StuI-EcoRI fragment of pFHDEL1. The resultant plasmid pDBDF5 (Fig. 8) includes the promoter of EP-A-258 067 to direct the ex-

pression of the HSA secretion signal fused to DNA encoding amino acids 1-387 of mature HSA, in turn fused directly and in frame with DNA encoding amino acids 585-1578 of human fibronectin, after which translation would terminate at the stop codon TAA. This is then followed by the *S.cerevisiae* PGK gene transcription terminator. The plasmid also contains sequences which permit selection and maintenance in *Escherichia coli* and *S.cerevisiae* (EP-A-258 067).

This plasmid was introduced into *S.cerevisiae* S150-2B (*leu2-3 leu2-112 ura3-52 trp1-289 his3-1*) by standard procedures (Beggs, 1978). Transformants were subsequently analysed and found to produce the HSA-fibronectin fusion protein.

EXAMPLE 2 : HSA 1-195 FUSED TO Fn 585-1578

In this second example the first domain of human serum albumin (amino acids 1-195) is fused to amino acids 585-1578 of human fibronectin.

The plasmid pDBD2 was digested with *Bam*HI and *Bgl*II and the 0.79kb fragment was purified and then ligated with *Bam*HI-digested M13mp19 to form pDBDF6 (Fig. 6). The following oligonucleotide:

5'-C C A A A G C T C G A G G A A C T T C G-3'

was used as a mutagenic primer to create a *Xho*I site in pDBDF6 by *in vitro* mutagenesis using a kit supplied by Amersham International PLC. This site was created by changing base number 696 of HSA from a T to a G (Fig. 2). The plasmid thus formed was designated pDBDF7 (Fig. 9). The following linker was then synthesised to represent from this newly created *Xho*I site to the codon for lysine 195 of HSA (AAA) and then from the codon for isoleucine 585 of fibronectin to the ends of oligonucleotides 1 and 8 shown in Fig. 6.

Linker 7

D E L R D E G K A S S A K
 TC GAT GAA CTT CGG GAT GAA GGG AAG GCT TCG TCT GCC AAA
 A CTT GAA GCC CTA CTT CCC TTC CGA AGC AGA CGG TTT
 I T E T P S Q P N S H
 ATC ACT GAG ACT CCG AGT CAG C
 TAG TGA CTC TGA GGC TCA GTC GGG TTG AGG GTG G

This linker was ligated with the annealed oligonucleotides shown in Fig. 3, i.e. 2+7, 3+6 and 4+5 together with *Xho*I and *Eco*RI digested pDBDF7 to form pDBDF8 (Fig. 9). Note that in order to recreate the original HSA DNA sequence, and hence amino acid sequence, insertion of linker 7 and the other oligonucleotides into pDBDF7 does not recreate the *Xho*I site.

The 0.83kb *Bam*HI-*Stu*I fragment of pDBDF8 was purified and then was ligated with the 0.68kb *Eco*RI-*Bam*HI fragment of pDBDF2 and the 2.22kb *Stu*I-*Eco*RI fragment of pFHDEL1 into *Bgl*II-digested pKV50 to form pDBDF9 (Fig. 9). This plasmid is similar to pDBDF5 except that it specifies only residues 1-195 of HSA rather than 1-387 as in pDBDF5.

When introduced into *S.cerevisiae* S150-2B as above, the plasmid directed the expression and secretion of a hybrid molecule composed of residues 1-195 of HSA fused to residues 585-1578 of fibronectin.

EXAMPLE 3 : HSA 1-387 FUSED TO Fn 585-1578, AS CLEAVABLE MOLECULE

In order to facilitate production of large amounts of residues 585-1578 of fibronectin, a construct was made in which DNA encoding residues 1-387 of HSA was separated from DNA encoding residues 585-1578 of fibronectin by the sequence

I E G R
 ATT GAA GGT AGA
 TAA CTT CCA TCT

5

which specifies the cleavage recognition site for the blood clotting Factor X. Consequently the purified secreted product can be treated with Factor X and then the fibronectin part of the molecule can be separated from the HSA part.

10

To do this two oligonucleotides were synthesised and then annealed to form Linker 8.

Linker 8

15

E E P Q N L I E G
 GAA GAG CCT CAG AAT TTA ATT GAA GGT
 CTT CTC GGA GTC TTA AAT TAA CTT CCA

20

R I T E T P S Q P
 AGA ATC ACT GAG ACT CCG AGT CAG C
 TCT TAG TGA CTC TGA GGC TCA GTC GGG

25

30

N S H

35

TTG AGG GTG G

This linker was then ligated with the annealed oligonucleotides shown in Fig. 6, i.e. 2+7, 3+6 and 4+5 into HincII and EcoRI digested mHOB12, to form pDBDF10 (Fig. 7). The plasmid was then digested with PstI and EcoRI and the roughly 0.24kb fragment was purified and then ligated with the 1.29kb BamHI-PstI fragment of pDBD2 and BamHI and EcoRI digested pUC19 to form pDBDF11 (Fig. 10).

40

The 1.5kb BamHI-StuI fragment of pDBDF11 was then ligated with the 0.68kb EcoRI-BamHI fragment of pDBDF4 and the 2.22kb StuI-EcoRI fragment of pFHDEL1 into BglII-digested pKV50 to form pDBDF12 (Fig. 10). This plasmid was then introduced into *S.cerevisiae* S150-2B. The purified secreted fusion protein was treated with Factor X to liberate the fibronectin fragment representing residues 585-1578 of the native molecule.

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Claims

Claims for the following Contracting States : AT, BE, CH, LI, DE, DK, FR, IT, LU, NL, SE

- 5 1. A fusion polypeptide comprising, as at least part of the N-terminal portion thereof, an N-terminal portion of HSA or a variant thereof and, as at least part of the C-terminal portion thereof, another polypeptide except that, when the said N-terminal portion of HSA is the 1-n portion where n is 369 to 419 or a variant thereof then the said polypeptide is (a) the 585 to 1578 portion of human fibronectin or a variant thereof, (b) the 1 to 368 portion of CD4 or a variant thereof, (c) platelet derived growth factor or a variant thereof, 10 (d) transforming growth factor β or a variant thereof, (e) the 1-261 portion of mature human plasma fibronectin or a variant thereof, (f) the 278-578 portion of mature human plasma fibronectin or a variant thereof, (g) the 1-272 portion of mature human von Willebrand's Factor or a variant thereof, or (h) alpha-1-antitrypsin or a variant thereof.
- 15 2. A fusion polypeptide according to Claim 1 additionally comprising at least one N-terminal amino acid extending beyond the portion corresponding to the N-terminal portion of HSA.
3. A fusion polypeptide according to Claim 1 or 2 wherein there is a cleavable region at the junction of the said N-terminal or C-terminal portions.
- 20 4. A fusion polypeptide according to any one of the preceding claims wherein the said C-terminal portion is the 585 to 1578 portion of human plasma fibronectin or a variant thereof.
5. A transformed or transfected host having a nucleotide sequence so arranged as to express a fusion polypeptide according to any one of the preceding claims.
- 25 6. A process for preparing a fusion polypeptide by cultivation of a host according to Claim 5, followed by separation of the fusion polypeptide in a useful form.
7. A fusion polypeptide according to any one of Claims 1 to 4 for use in therapy.

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Claims for the following Contracting States : ES, GR

- 35 1. A process for preparing a fusion polypeptide by (i) cultivation of a transformed or transfected host having a nucleotide sequence so arranged as to express a fusion polypeptide, followed by (ii) separation of the fusion polypeptide in a useful form, characterised in that the fusion polypeptide comprises as at least part of the N-terminal portion thereof, an N-terminal portion of HSA or a variant thereof and, as at least part of the C-terminal portion thereof, another polypeptide except that, when the said N-terminal portion of HSA is the 1-n portion where n is 369 to 419 or a variant thereof then the said polypeptide is (a) the 585 to 1578 portion of human fibronectin or a variant thereof, (b) the 1 to 368 portion of CD4 or a variant thereof, 40 (c) platelet derived growth factor or a variant thereof, (d) transforming growth factor β or a variant thereof, (e) the 1-261 portion of mature human plasma fibronectin or a variant thereof, (f) the 278-578 portion of mature human plasma fibronectin or a variant thereof, (g) the 1-272 portion of mature human von Willebrand's Factor or a variant thereof, or (h) alpha-1-antitrypsin or a variant thereof.
- 45 2. A process according to Claim 1, wherein the fusion polypeptide additionally comprising at least one N-terminal amino acid extending beyond the portion corresponding to the N-terminal portion of HSA.
3. A process according to Claim 1 or 2 wherein, in the fusion polypeptide, there is a cleavable region at the junction of the said N-terminal or C-terminal portions.
- 50 4. A process according to any one of the preceding claims wherein the said C-terminal portion is the 585 to 1578 portion of human plasma fibronectin or a variant thereof.

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Patentansprüche

Patentansprüche für folgende Vertragsstaaten : AT, BE, CH, DE, DK, FR, IT, LU, NL, SE

- 5 1. Fusionspolypeptid, umfassend als mindestens einen Teil seines N-terminalen Teils einen N-terminalen Teil von HSA oder eine Variante davon und als mindestens einen Teil seines C-terminalen Teils ein weiteres Polypeptid mit der Ausnahme, daß wenn es sich bei dem N-terminalen Teil von HSA um den Teil 1-n mit $n = 369$ bis 419 oder eine Variante davon handelt, das Polypeptid aus
 - 10 (a) dem Teil 585 bis 1578 von Humanfibronectin oder einer Variante davon,
 - (b) dem Teil 1 bis 368 von CD4 oder einer Variante davon,
 - (c) dem "Platelet Derived Growth Factor" (PDGF) oder einer Variante davon,
 - (d) dem "Transforming Growth Factor β " (TGF β) oder einer Variante davon,
 - (e) dem Teil 1-261 von reifem Humanplasmafibronektin oder einer Variante davon,
 - (f) dem Teil 278-578 von reifem Humanplasmafibronektin oder einer Variante davon,
 - 15 (g) dem Teil 1-272 von reifem Human-von Willebrand's-Faktor oder einer Variante davon oder
 - (h) Alpha-1-Antitrypsin oder einer Variante davon, besteht.
2. Fusionspolypeptid nach Anspruch 1, zusätzlich umfassend mindestens eine N-terminale Aminosäure, die länger als der dem N-terminalen Teil von HSA entsprechende Teil ist.
- 20 3. Fusionspolypeptid nach Anspruch 1 oder 2, bei dem sich an der Verbindung der N-terminalen oder C-terminalen Teile eine spaltbare Region befindet.
4. Fusionspolypeptid nach einem der vorhergehenden Ansprüche, wobei der C-terminale Teil aus dem Teil 585 bis 1578 von Humanplasmafibronektin oder einer Variante davon besteht.
- 25 5. Transformierter oder transfizierter Wirt mit einer Nukleotidsequenz, die so angeordnet ist, daß sie ein Fusionspolypeptid nach einem der vorhergehenden Ansprüche exprimieren kann.
6. Verfahren zur Herstellung eines Fusionspolypeptids durch Kultivieren eines Wirts nach Anspruch 5 und anschließendes Abtrennen des Fusionspolypeptids in einer geeigneten Form.
- 30 7. Fusionspolypeptid nach einem der Ansprüche 1 bis 4 zur therapeutischen Verwendung.

Patentansprüche für folgende Vertragsstaaten : ES, GR

- 35 1. Verfahren zur Herstellung eines Fusionspolypeptids durch
 - (i) Kultivieren eines transformierten oder transfizierten Wirts mit einer Nukleotidsequenz, die so angeordnet ist, daß sie ein Fusionspolypeptid exprimiert, und
 - (ii) anschließendes Abtrennen des Fusionspolypeptids in einer geeigneten Form,
 - 40 dadurch gekennzeichnet, daß das Fusionspolypeptid als mindestens einen Teil seines N-terminalen Teils einen N-terminalen Teil von HSA oder eine Variante davon und als mindestens einen Teil seines C-terminalen Teils ein weiteres Polypeptid umfaßt, mit der Ausnahme, daß wenn es sich bei dem N-terminalen Teil von HSA um den Teil 1-n mit $n = 369$ bis 419 oder eine Variante davon handelt, das Polypeptid aus
 - 45 (a) dem Teil 585-1578 von Humanfibronectin oder einer Variante davon,
 - (b) dem Teil 1-368 von CD4 oder einer Variante davon,
 - (c) dem Platelet Derived Growth Factor oder einer Variante davon,
 - (d) dem Transforming Growth Factor β oder einer Variante davon,
 - (e) dem Teil 1-261 von reifem Humanplasmafibronektin oder einer Variante davon,
 - (f) dem Teil 278-578 von reifem Humanplasmafibronektin oder einer Variante davon,
 - 50 (g) dem Teil 1-272 von reifem Human-von Willebrand's-Faktor oder einer Variante davon oder
 - (h) α -1-Antitrypsin oder einer Variante davon besteht.
2. Verfahren nach Anspruch 1, wobei das Fusionspolypeptid zusätzlich mindestens eine N-terminale Aminosäure, die länger als der dem N-terminalen Teil von HSA entsprechende Teil ist, umfaßt.
- 55 3. Verfahren nach Anspruch 1 oder 2, wobei sich in dem Fusionspolypeptid an der Verbindung der N-terminalen oder C-terminalen Teile eine spaltbare Region befindet.

4. Verfahren nach einem der vorhergehenden Ansprüche, wobei der C-terminale Teil aus dem Teil 585-1578 von Humanplasmafibronectin oder einer Variante davon besteht.

5 Revendications

Revendications pour les Etats contractants suivants : AT, BE, CH, DE, DK, FR, IT, LU, NL, SE

- 10 1. Polypeptide fusionné comprenant en tant qu'au moins une partie de sa portion N-terminale, une portion N-terminale de HSA ou d'un variant de celle-ci et, en tant qu'au moins une partie de sa portion C-terminale, un autre polypeptide sauf que, lorsque cette portion N-terminale de HSA est la portion 1-n dans laquelle n est 369 à 419 ou un variant de celle-ci, ce polypeptide est (a) la portion 585 à 1578 de la fibronectine humaine ou un variant de celle-ci, (b) la portion 1 à 368 de CD4 ou un variant de celle-ci, (c) le facteur de croissance dérivé des plaquettes sanguines ou un variant de celui-ci, (d) le facteur de croissance β de transformation ou un variant de celui-ci, (e) la portion 1-261 de la fibronectine mature de plasma humain ou un variant de celle-ci, (f) la portion 278-578 de la fibronectine mature de plasma humain ou un variant de celle-ci, (g) la portion 1-272 du facteur humain mature de von Willebrand ou un variant de celle-ci, ou (h) l'alpha-1-antitrypsine ou un variant de celle-ci.
- 15 2. Polypeptide fusionné suivant la revendication 1, comprenant de plus au moins un acide aminé N-terminal se prolongeant au-delà de la portion correspondant à la portion N-terminale de HSA.
- 20 3. Polypeptide fusionné suivant les revendications 1 ou 2, dans lequel il y a une région susceptible d'être coupée à la jonction de ces portions N-terminale et C-terminale.
- 25 4. Polypeptide fusionné suivant l'une quelconque des revendications précédentes, dans lequel cette portion C-terminale est la portion 585 à 1578 de la fibronectine de plasma humain ou un variant de celle-ci.
- 30 5. Hôte transformé ou transfecté ayant une séquence de nucléotides arrangée de façon à exprimer un polypeptide fusionné suivant l'une quelconque des revendications précédentes.
6. Procédé pour préparer un polypeptide fusionné par culture d'un hôte suivant la revendication 5, suivie de la séparation du polypeptide fusionné sous une forme utile.
- 35 7. Polypeptide fusionné suivant l'une quelconque des revendications 1 à 4 utilisable en thérapie.

Revendications pour les Etats contractants suivants : ES, GR

- 40 1. Procédé pour préparer un polypeptide fusionné par (i) la culture d'un hôte transformé ou transfecté ayant une séquence de nucléotides arrangée de façon à exprimer un polypeptide fusionné, suivie de (ii) la séparation du polypeptide fusionné sous une forme utile, caractérisé en ce que le polypeptide fusionné comprend, en tant qu'au moins une partie de sa portion N-terminale, une portion N-terminale de HSA ou d'un variant de celle-ci et, en tant qu'au moins une partie de sa portion C-terminale, un autre polypeptide sauf que, lorsque cette portion N-terminale de HSA est la portion 1-n dans laquelle n est 369 à 419 ou un variant de celle-ci, ce polypeptide est alors (a) la portion 585 à 1578 de la fibronectine humaine ou un variant de celle-ci, (b) la portion 1 à 368 de CD4 ou un variant de celle-ci, (c) le facteur de croissance dérivé des plaquettes sanguines ou un variant de celui-ci, (d) le facteur de croissance β de transformation ou un variant de celui-ci, (e) la portion 1-261 de la fibronectine mature de plasma humain ou un variant de celle-ci, (f) la portion 278-578 de la fibronectine mature de plasma humain ou un variant de celle-ci, (g) la portion 1-272 du facteur humain mature de von Willebrand ou un variant de celle-ci, ou (h) l'alpha-1-antitrypsine ou un variant de celle-ci.
- 45 2. Procédé suivant la revendication 1, dans lequel le polypeptide fusionné comprend de plus au moins un acide aminé N-terminal se prolongeant au-delà de la portion correspondant à la portion N-terminale de HSA.
- 50 3. Procédé suivant les revendications 1 ou 2 dans lequel, dans le polypeptide fusionné, il y a une région susceptible d'être coupée à la jonction de ces portions N-terminale et C-terminale.
- 55

4. Procédé suivant l'une quelconque des revendications précédentes, dans lequel cette portion C-terminale est la portion 585 à 1578 de la fibronectine de plasma humain ou un variant de celle-ci.

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FIGURE 1

10	20
Asp Ala His Lys Ser Glu Val Ala His Arg Phe Lys Asp Leu Gly Glu Glu Asn Phe Lys	
30	40
Ala Leu Val Leu Ile Ala Phe Ala Gln Tyr Leu Gln Gln Cys Pro Phe Glu Asp His Val	
50	60
Lys Leu Val Asn Glu Val Thr Glu Phe Ala Lys Thr Cys Val Ala Asp Glu Ser Ala Glu	
70	80
Asn Cys Asp Lys Ser Leu His Thr Leu Phe Gly Asp Lys Leu Cys Thr Val Ala Thr Leu	
90	100
Arg Glu Thr Tyr Gly Glu Met Ala Asp Cys Cys Ala Lys Gln Glu Pro Glu Arg Asn Glu	
110	120
Cys Phe Leu Gln His Lys Asp Asp Asn Pro Asn Leu Pro Arg Leu Val Arg Pro Glu Val	
130	140
Asp Val Met Cys Thr Ala Phe His Asp Asn Glu Glu Thr Phe Leu Lys Lys Tyr Leu Tyr	
150	160
Glu Ile Ala Arg Arg His Pro Tyr Phe Tyr Ala Pro Glu Leu Leu Phe Phe Ala Lys Arg	
170	180
Tyr Lys Ala Ala Phe Thr Glu Cys Cys Gln Ala Ala Asp Lys Ala Ala Cys Leu Leu Pro	
190	200
Lys Leu Asp Glu Leu Arg Asp Glu Gly Lys Ala Ser Ser Ala Lys Gln Arg Leu Lys Cys	
210	220
Ala Ser Leu Gln Lys Phe Gly Glu Arg Ala Phe Lys Ala Trp Ala Val Ala Arg Leu Ser	
230	240
Gln Arg Phe Pro Lys Ala Glu Phe Ala Glu Val Ser Lys Leu Val Thr Asp Leu Thr Lys	
250	250
Val His Thr Glu Cys Cys His Gly Asp Leu Leu Glu Cys Ala Asp Asp Arg Ala Asp Leu	
270	280
Ala Lys Tyr Ile Cys Glu Asn Gln Asp Ser Ile Ser Ser Lys Leu Lys Glu Cys Cys Glu	
290	300
Lys Pro Leu Leu Glu Lys Ser His Cys Ile Ala Glu Val Glu Asn Asp Glu Met Pro Ala	
310	320
Asp Leu Pro Ser Leu Ala Ala Asp Phe Val Glu Ser Lys Asp Val Cys Lys Asn Tyr Ala	
330	340
Glu Ala Lys Asp Val Phe Leu Gly Met Phe Leu Tyr Glu Tyr Ala Arg Arg His Pro Asp	
350	360
Tyr Ser Val Val Leu Leu Leu Arg Leu Ala Lys Thr Tyr Glu Thr Thr Leu Glu Lys Cys	
370	380
Cys Ala Ala Ala Asp Pro His Glu Cys Tyr Ala Lys Val Phe Asp Glu Phe Lys Pro Leu	

FIGURE 1 Cont.

	390	400
Val Glu Glu Pro Gln Asn Leu Ile Lys Gln Asn Cys Glu Leu Phe Glu Gln Leu Gly Glu		
	410	420
Tyr Lys Phe Gln Asn Ala Leu Leu Val Arg Tyr Thr Lys Lys Val Pro Gln Val Ser		Thr
	430	440
Pro Thr Leu Val Glu Val Ser Arg Asn Leu Gly Lys Val Gly Ser Lys Cys Cys Lys His		
	450	460
Pro Glu Ala Lys Arg Met Pro Cys Ala Glu Asp Tyr Leu Ser Val Val Leu Asn Gln Leu		
	470	480
Cys Val Leu His Glu Lys Thr Pro Val Ser Asp Arg Val Thr Lys Cys Cys Thr Glu Ser		
	490	500
Leu Val Asn Arg Arg Pro Cys Phe Ser Ala Leu Glu Val Asp Glu Thr Tyr Val Pro Lys		
	510	520
Glu Phe Asn Ala Glu Thr Phe Thr Phe His Ala Asp Ile Cys Thr Leu Ser Glu Lys Glu		
	530	540
Arg Gln Ile Lys Lys Gln Thr Ala Leu Val Glu Leu Val Lys His Lys Pro Lys Ala Thr		
	550	560
Lys Glu Gln Leu Lys Ala Val Met Asp Asp Phe Ala Ala Phe Val Glu Lys Cys Cys Lys		
	570	580
Ala Asp Asp Lys Glu Thr Cys Phe Ala Glu Glu Gly Lys Lys Leu Val Ala Ala Ser Gln		
Ala Ala Leu Gly Leu		

FIGURE 2 DNA sequence coding for mature HSA

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      10      20      30      40      50      60      70      80
GATGCACACAAGAGTGAGGTTGCTCATCGGTTTAAAGATTTGGGAGAAGAAAATTTCAAAGCCTTGGTGTGATTGCCTT
D A H K S E V A H R F K D L G E E N F K A L V L I A F

      90     100     110     120     130     140     150     160
TGCTCAGTATCTTCAGCAGTGTCCATTTGAAGATCATGTAAAATTAGTGAATGAAGTAACTGAATTTGCAAAAACATGTG
A Q Y L Q Q C F F E D H V K L V N E V T E F A K T C

      170     180     190     200     210     220     230     240
TGGCTGATGAGTTCAGCTGAAAATTTGTGACAAATCACTTCATACCCCTTTTGGAGACAAATTATGCACAGTTGCAACTCTT
V A D E S A E N C D K S L H T L F G D K L C T V A T L

      250     260     270     280     290     300     310     320
CGTGAAACCTATGGTGAAATGGCTGACTGCTGTGCAAAACAAGAACCTGAGAGAAATGAATGCTTCTTGCAACACAAAGA
R E T Y G E M A D C C A K Q E P E R N E C F L Q H K D

      330     340     350     360     370     380     390     400
TGACAACCCAAACCTCCCCCGATTGGTGAGACCAAGAGTTGATGTGATGTGCACTGCTTTTCATGACAATGAAGAGACAT
D N P N L P R L V R P E V D V M C T A F H D N E E T

      410     420     430     440     450     460     470     480
TTTGTAAAAAATACTTATATGAAATTCGACAGAAGACATCCTTACTTTTATGCCCGGAACTCCTTTTCTTGCTAAAAGG
F L K K Y L Y E I A R R H P Y F Y A P E L L F F A K R

      490     500     510     520     530     540     550     560
TATAAAGCTGCTTTTACAGAATGTGTGCAAGCTGCTGATAAAGCTGCCTGCCTGTTGCCAAAGCTCGATGAACCTTCGGGA
Y K A A F T E C C Q A A D K A A C L L P K L D E L R D

      570     580     590     600     610     620     630     640
TGAAGGGAAGGCTTCGTCTGCCAAACAGAGACTCAATGTGCCAGTCTCCAAAAATTTGGAGAAAGAGCTTTCAAGCAT
E G K A S S A K Q R L K C A S L Q K F G E R A F K A

      650     660     670     680     690     700     710     720
GGGCAGTGGCTCGCCTGAGCCAGAGATTTCCCAAAGCTGAGTTTGCAGAAGTTTCCAAGTTAGTGACAGATCTTACCAA
W A V A R L S Q R F P K A E F A E V S K L V T D L T K

      730     740     750     760     770     780     790     800
GTCCACACGGAATGCTGCCATGGAGATCTGCTTGAATGTGCTGATGACAGGGCGGACCTTGCCAACTATATCTGTGAAAA
V L T E C C H G D L L E C A D D R A D L A K Y I C E N

      810     820     830     840     850     860     870     880
TCAGGATTGATCTCCAGTAAACTGAAGGAATGCTGTGAAAAACCTCTGTTGGAAAAATCCCACTGCATTGCCGAAGTGG
Q D S I S S K L K E C C E K P L L E K S H C I A E V

      890     900     910     920     930     940     950     960
AAAATGATGAGATGCGCTGCTGACTTGCTTCATTAGCTGCTGATTTTGTGAAAGTAAGGATGTTTGCAAAAACCTATGCT
E N D E M P A D L P S L A A D F V E S K D V C K N Y A

      970     980     990     1000     1010     1020     1030     1040
GAGGCAAAGGATGTCTTCTGGGCATGTTTTTGTATGAATATGCAAGAAGGCATCCTGATTACTCTGTCTGCTGCTGCT
E A K D V F L G M F L Y E Y A R R H P D Y S V V L L L

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FIGURE 2 Cont.

1050 1060 1070 1080 1090 1100 1110 1120
 GAGACTTGCCAAAGACATATGAAACCACTCTAGAGAAAGTGTGTGCGCGCTGCAGATCCTCATGAATGCTATGCCAAAGTGT
 R L A K T Y E T T L E K C C A A A D P H E C Y A K V

1130 1140 1150 1160 1170 1180 1190 1200
 TCGATGAATTTAAACCTCTTGTGGAAGAGCCTCAGAATTTAATCAAACAAAACCTGTGAGCTTTTGGAGCAGCTTGGAGAG
 F D E F K P L V E E P Q N L I K Q N C E L F E Q L G E

1210 1220 1230 1240 1250 1260 1270 1280
 TACAATTCAGAAATGGCTATTAGTTCTGTTACACCAAGAAAGTACCCCAAGTGTCAACTCCAACCTCTGTAGAGGTCTC
 Y K F Q N A L L V R Y T K K V P Q V S T P T L V E V S

1290 1300 1310 1320 1330 1340 1350 1360
 AAGAAACCTAGGAAAAGTGGGCAGCAAAATGTTGTAACATCCTGAAGCAAAAAGAATGCCCTGTGCAGAAAGACTATCTAT
 R N L G K V G S K C C K H P E A K R M P C A E D Y L

1370 1380 1390 1400 1410 1420 1430 1440
 CCGTGGTCTGTAACCAAGTATTGTGTGTGTGCATGAGAAAACGCCAGTAAGTGACAGAGTCACAAAATGCTGCACAGAGTCC
 S V V L N Q L C V L H E K T P V S D R V T K C C T E S

1450 1460 1470 1480 1490 1500 1510 1520
 TTGGTGAACAGGCGACCATGCTTTTCAGCTCTGGAAGTCGATGAAACATACGTTCCCAAAGAGTTTAAATGCTGAAACATT
 L V N R R P C F S A L E V D E T Y V P K E F N A E T F

1530 1540 1550 1560 1570 1580 1590 1600
 CACCTTCATGCAGATATATGCACACTTTCTGAGAAGGAGAGACAAATCAAGAAACAAACTGCACCTTGTGAGCTTGTGA
 T F H A D I C T L S E K E R Q I K K Q T A L V E L V

1610 1620 1630 1640 1650 1660 1670 1680
 AACACAAGCCCAAGGCAACAAAAGAGCAACTGAAAGCTGTTATGGATGATTTCCGAGCTTTTGTAGAGAAGTGTGCAAG
 K H K P K A T K E Q L K A V M D D F A A F V E K C C K

1690 1700 1710 1720 1730 1740 1750 1760
 GCTGACGATAAGGAGACCTGCTTTGCCGAGGAGGGTAAAAAACTGTGCTGCAAGTCAAGCTGCCCTTAGGCTTATAACA
 A D D K E T C F A E E G K K L V A A S Q A A L G L

1770 1780
 TCTACATTTAAAAGCATCTCAG

FIGURE 3 Construction of mHOB16

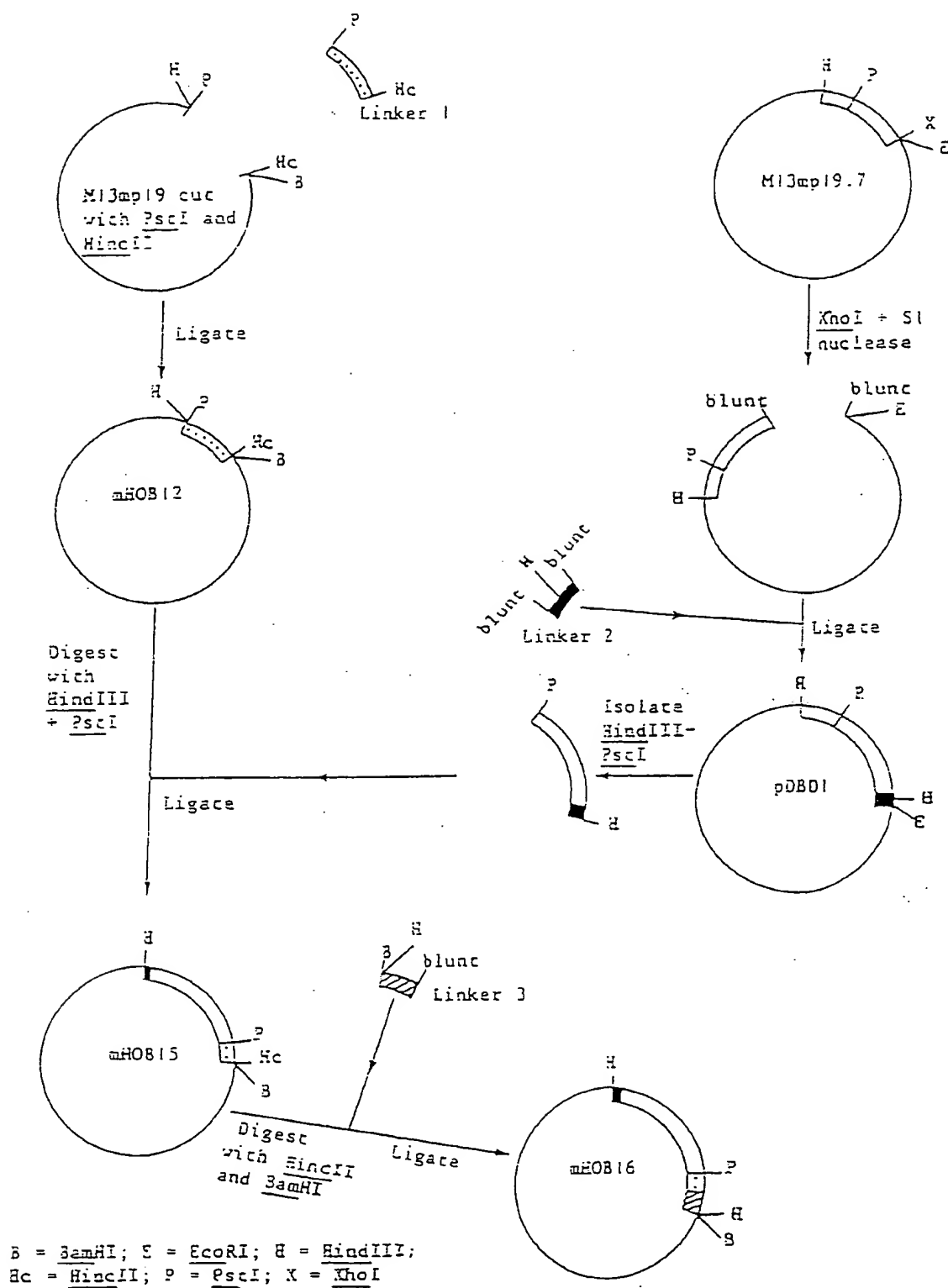
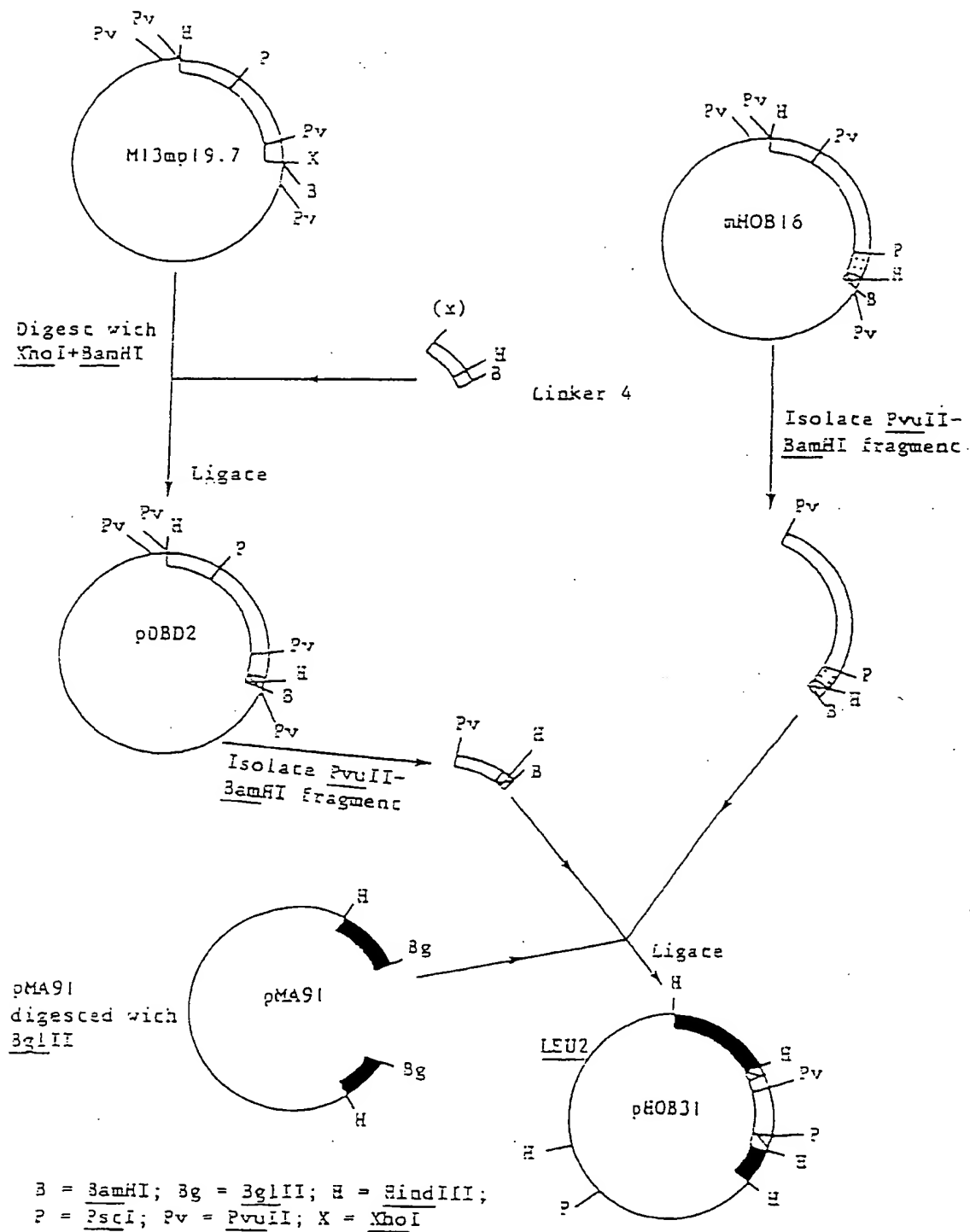


FIGURE 4 Construction of pEOB31



Gln Ala Gln Gln Met Val Gln Pro Gln Ser¹⁰ Pro Val Ala Val Ser Gln Ser Lys Pro Gly²⁰
 Cys Tyr Asp Asn Gly Lys His Tyr Gln Ile³⁰ Asn Gln Gln Trp Glu Arg Thr Tyr Leu Gly⁴⁰
 Asn Val Leu Val Cys Thr Cys Tyr Gly Gly Ser⁵⁰ Arg Gly Phe Asn Cys Glu Ser Lys Pro⁶⁰
 Glu Ala Glu Glu Thr Cys Phe Asp Lys Tyr⁷⁰ Thr Gly Asn Thr Tyr Arg Val Gly Asp Thr⁸⁰
 Tyr Glu Arg Pro Lys Asp Ser Met Ile Trp⁹⁰ Asp Cys Thr Cys Ile Gly Ala Gly Arg Gly¹⁰⁰
 Arg Ile Ser Cys Thr Ile Ala Asn Arg Cys¹¹⁰ His Glu Gly Gly Gln Ser Tyr Lys Ile Gly¹²⁰
 Asp Thr Trp Arg Arg Pro His Glu Thr Gly¹³⁰ Gly Tyr Met Leu Glu Cys Val Cys Leu Gly¹⁴⁰
 Asn Gly Lys Gly Glu Trp Thr Cys Lys Pro¹⁵⁰ Ile Ala Glu Lys Cys Phe Asp His Ala Ala¹⁶⁰
 Gly Thr Ser Tyr Val Val Gly Glu Thr¹⁷⁰ Trp Glu Lys Pro Tyr Gln Gly Trp Met Met Val¹⁸⁰
 Asp Cys Thr Cys Leu Gly Glu Gly Ser¹⁹⁰ Arg Ile Thr Cys Thr Ser Arg Asn Arg Cys²⁰⁰
 Asn Asp Gln Asp Thr Arg Thr Ser Tyr²¹⁰ Arg Ile Gly Asp Thr Trp Ser Lys Lys Asp Asn²²⁰
 Arg Gly Asn Leu Leu Gln Cys Ile Cys Thr²³⁰ Gly Asn Gly Arg Gly Glu Trp Lys Cys Glu²⁴⁰
 Arg His Thr Ser Val Gln Thr Thr Ser²⁵⁰ Gly Ser Gly Pro Phe Thr Asp Val Arg Ala²⁶⁰
 Ala Val Tyr Gln Pro Gln Pro His Pro²⁷⁰ Gln Pro Pro Tyr Gly His Cys Val Thr Asp²⁸⁰
 Ser Ely Val Val Tyr Ser Val Gly Met²⁹⁰ Gln Trp Leu Lys Thr Gln Gly Asn Lys Gln Met³⁰⁰
 Leu Cys Thr Cys Leu Gly Asn Gly Val³¹⁰ Ser Cys Gln Glu Thr Ala Val Thr Gln Thr Tyr³²⁰
 Gly Gly Asn Ser Asn Gly Glu Pro Cys³³⁰ Val Leu Pro Phe Thr Tyr Asn Gly Arg Thr Phe³⁴⁰
 Tyr Ser Cys Thr Thr Glu Gly Arg Gln³⁵⁰ Asp Gly His Leu Trp Cys Ser Thr Thr Ser Asn³⁶⁰
 Tyr Glu Gln Asp Gln Lys Tyr Ser³⁷⁰ Phe Cys Thr Asp His Thr Val Leu Val Gln Thr Gln³⁸⁰
 Gly Gly Asn Ser Asn Ely Ala Leu Cys³⁹⁰ His Phe Pro Phe Leu Tyr Asn Asn His Asn Tyr⁴⁰⁰
 Thr Asp Cys Thr Ser Glu Gly Arg Arg⁴¹⁰ Asp Asn Met Lys Trp Cys Gly Thr Thr Gln Asn⁴²⁰

Fig. 5A

Tyr Asp Ala Asp Gln Lys Phe Gly Phe Cys 430 Pro Met Ala Ala His Glu Glu Ile Cys 440 Thr
 Thr Asn Glu Gly Val Met Tyr Arg Ile Gly Asp Gln Trp Asp Lys Gln His Asp Met Gly 450
 His Met Met Arg Cys Thr Cys Val Gly Asn Gly Arg Gly Glu Trp Thr Cys Tyr Ala Tyr 460
 Ser Gln Leu Arg Asp Gln Cys Ile Val Asp Asp Ile Thr Tyr Asn Val Asn Asp Thr 470
 His Lys Arg His Glu Glu Gly His Met Leu Asn Cys Thr Cys Phe Gly Gln Gly Arg 480
 Arg Trp Lys Cys Asp Pro Val Asp Gln Cys Gln Asp Ser Glu Thr Gly Thr Phe Tyr 490
 Ile Gly Asp Ser Trp Glu Lys Tyr Val His Gly Val Arg Tyr Gln Cys Tyr Cys Tyr 500
 Arg Gly Ile Gly Glu Trp His Cys Gln Pro Leu Gln Thr Tyr Pro Ser Ser Ser Gly 510
 Val Glu Val Phe Ile Thr Glu Thr Pro Ser Gln Pro Asn Ser His Pro Ile Gln Trp Asn 520
 Ala Pro Gln Pro Ser His Ile Ser Lys Tyr Ile Leu Arg Trp Arg Pro Lys Asn Ser 530
 Gly Arg Trp Lys Glu Ala Thr Ile Pro Gly His Leu Asn Ser Tyr Thr Ile Lys Gly 540
 Lys Pro Gly Val Val Tyr Glu Gly Gln Leu Ile Ser Ile Gln Gln Tyr Gly His Gln 550
 Val Thr Arg Phe Asp Phe Thr Thr Thr Ser Thr Ser Thr Pro Val Thr Ser Asn Thr 560
 Thr Gly Glu Thr Thr Pro Phe Ser Pro Leu Val Ala Thr Ser Glu Ser Val Thr Glu 570
 Thr Ala Ser Ser Phe Val Val Ser Trp Val Ser Ala Ser Asp Thr Val Ser Gly Phe Arg 580
 Val Glu Tyr Glu Leu Ser Glu Gly Asp Glu Pro Gln Tyr Leu Asp Leu Pro Ser Thr 590
 Ala Thr Ser Val Asn Ile Pro Asp Leu Leu Pro Ely Arg Lys Tyr Ile Val Asn Val Tyr 600
 Gln Ile Ser Glu Asp Gly Glu Gln Ser Leu Ile Leu Ser Thr Ser Gln Thr Thr Ala 610
 Asp Ala Pro Pro Asp Pro Thr Val Asp Gln Val Asp Asp Thr Ser Ile Val Val Arg Trp 620
 Ser Arg Pro Gln Ala Pro Ile Thr Gly Tyr Arg Ile Val Tyr Ser Pro Ser Val Glu Gly 630
 Ser Ser Thr Glu Leu Asn Leu Pro Glu Thr Ala Asn Ser Val Thr Leu Ser Asp Leu Gln 640
 FNDEL 1

Fig. 5B

Pro Gly Val Gln Tyr Asn Ile Thr Ile Tyr Ala Val Glu Glu Asn Gln Glu Ser Thr 850
 Val Val Ile Gln Gln Glu Thr Thr Gly 870 Thr Pro Arg Ser Asp Thr Val Pro Ser Pro Arg 880
 Aso Leu Gln Phe Val Glu Val Thr Asp Val Lys Val Thr Ile Met Trp Thr Pro Pro Glu 900
 Ser Ala Val Thr Gly Tyr Arg Val Asp Val Ile Pro Val Asn Leu Pro Gly Glu His Gly 920
 Gln Arg Leu Pro Ile Ser Arg Asn Thr Phe Ala Glu Val Thr Gly Leu Ser Pro Gly Val 940
 Thr Tyr Tyr Phe Lys Val Phe Ala Val Ser His Gly Arg Glu Ser Lys Pro Leu Thr Ala 960
 Gln Gln Thr Thr Lys Leu Asp Ala Pro Thr Asn Leu Gln Phe Val Asn Glu Thr Asp Ser 980
 Thr Val Leu Val Arg Trp Thr Pro Pro Arg Ala Gln Ile Thr Gly Tyr Arg Leu Thr Val 1000
 Gly Leu Thr Arg Arg Gly Gln Pro Arg Gln Tyr Asn Val Gly Pro Ser Val Ser Lys Tyr 1020
 Pro Leu Arg Asn Leu Gln Pro Ala Ser Glu Tyr Thr Val Ser Leu Val Ala Ile Lys Gly 1040
 Asn Gln Glu Ser Pro Lys Ala Thr Gly Val Phe Thr Thr Leu Gln Pro Gly Ser Ser Ile 1060
 Pro Pro Tyr Asn Thr Glu Val Thr Glu Thr Thr Ile Val Ile Thr Trp Thr Pro Ala Pro 1080
 Arg Ile Gly Phe Lys Leu Gly Val Arg Pro Ser Gln Gly Gly Glu Ala Pro Arg Glu Val 1100
 Thr Ser Asp Ser Gly Ser Ile Val Val Ser Gly Leu Thr Thr Pro Gly Val Glu Tyr Val Tyr 1120
 Thr Ile Gln Val Leu Arg Asp Gly Gln Glu Arg Asp Ala Pro Ile Val Asn Lys Val Val 1140
 Thr Pro Leu Ser Pro Pro Thr Asn Leu His Leu Glu Ala Asn Pro Asp Thr Gly Val Leu 1160
 Thr Val Ser Trp Glu Arg Ser Thr Thr Pro Asp Ile Thr Gly Tyr Arg Ile Thr Thr Thr 1180
 Pro Thr Asn Gly Gln Gln Gly Asn Ser Leu Glu Glu Val Val His Ala Asp Gln Ser Ser 1200
 Cys Thr Phe Asp Asn Leu Ser Pro Gly Leu Glu Tyr Asn Val Ser Val Tyr Thr Val Lys 1220
 Asp Asp Lys Glu Ser Val Pro Ile Ser Asp Thr Ile Ile Pro Ala Val Pro Pro Thr 1240
 Asp Leu Arg Phe Thr Asn Ile Gly Pro Asp Thr Met Arg Val Thr Trp Ala Pro Pro Pro 1260

Fig. 5C

Ser Ile Asp Leu Thr Asn Phe Leu Val Arg Tyr Ser Pro Val Lys Asn Glu Glu Asp Val
 Ala Glu Leu Ser Ile Ser Pro Ser Asp Asn Ala Val Val Leu Thr Asn Leu Leu Pro Gly
 Thr Glu Tyr Val Val Ser Val Ser Val Tyr Glu Gln His Glu Ser Thr Pro Leu Arg
 Gly Arg Gln Lys Thr Gly Leu Asp Ser Pro Thr Gly Ile Asp Phe Ser Asp Ile Thr Ala
 Asn Ser Phe Thr Val His Trp Ile Ala Pro Arg Ala Thr Ile Thr Gly Tyr Arg Ile Arg
 His His Pro Glu His Phe Ser Gly Arg Pro Arg Glu Asp Arg Val Pro His Ser Arg Asn
 Ser Ile Thr Leu Thr Asn Leu Thr Pro Gly Thr Glu Tyr Val Val Ser Ile Val Ala Leu
 Asn Gly Arg Glu Glu Ser Pro Leu Leu Ile Gly Gln Gln Ser Thr Val Ser Asp Val Pro
 Arg Asp Leu Glu Val Val Ala Ala Thr Pro Thr Ser Leu Leu Ile Ser Trp Asp Ala Pro
 Ala Val Thr Val Arg Tyr Tyr Arg Ile Thr Tyr Gly Glu Thr Gly Gly Asn Ser Pro Val
 Gln Glu Phe Thr Val Pro Gly Ser Lys Ser Thr Ala Thr Ile Ser Gly Leu Lys Pro Gly
 Val Asp Tyr Thr Ile Thr Val Tyr Ala Val Thr Gly Arg Gly Asp Ser Pro Ala Ser Ser
 Lys Pro Ile Ser Ile Asn Tyr Arg Thr Glu Ile Asp Lys Pro Ser Gln Met Gln Val Thr
 Asp Val Gln Asp Asn Ser Ile Ser Val Lys Trp Leu Pro Ser Ser Pro Val Thr Gly
 Tyr Arg Val Thr Thr Thr Pro Lys Asn Gly Pro Gly Pro Thr Lys Thr Lys Thr Ala Gly
 Pro Asp Gln Thr Glu Met Thr Ile Glu Gly Leu Gln Pro Thr Val Glu Tyr Val Val Ser
 Val Tyr Ala Gln Asn Pro Ser Gly Glu Ser Gln Pro Leu Val Gln Thr Ala Val Thr Thr
 Ile Pro Ala Pro Thr Asp Leu Lys Phe Thr Gln Val Thr Pro Thr Ser Leu Ser Ala Gln
 Trp Thr Pro Pro Asn Val Gln Leu Thr Gly Tyr Arg Val Arg Val Thr Pro Lys Glu Lys
 Thr Gly Pro Met Lys Glu Ile Asn Leu Ala Pro Asp Ser Ser Val Val Val Ser Gly
 Leu Met Val Ala Thr Lys Tyr Glu Val Ser Val Tyr Ala Leu Lys Asp Thr Leu Thr Ser
 FNDEL 1

Fig. 5D

Arg Pro Ala Gln Gly Val Val Thr Thr Leu Glu Asn Val Ser Pro Pro Arg Arg Ala Arg 1690
 Val Thr Asp Ala Thr Glu Thr Thr Ile Thr Ile Ser Trp Arg Thr Lys Thr Glu Thr Ile 1710
 Thr Gly Phe Gln Val Asp Ala Val Pro Ala Asn Gly Gln Thr Pro Ile Gln Arg Thr Ile 1730
 Lys Pro Asp Val Arg Ser Tyr Thr Thr Gly Leu Gln Pro Gly Thr Asp Tyr Lys Ile 1750
 Tyr Leu Tyr Thr Lau Asn Asp Asn Ala Arg 1770
 Ala Ile Asp Ala Pro Ser Asn Lau Arg Phe 1790
 Ser Trp Gln Pro Pro Arg Ala Arg Ile Thr Gly Tyr Ile Ile Lys Tyr Glu Lys Pro Gly 1810
 Ser Pro Pro Arg Glu Val Val Pro Arg Pro Arg Pro Gly Val Thr Glu Ala Thr Ile Thr 1830
 Gly Leu Glu Pro Gly Thr Glu Tyr Thr Ile Tyr Val Ile Ala Leu Lys Asn Asn Gln Lys 1850
 Ser Glu Pro Leu Ile Gly Arg Lys Lys Thr 1870
 His Pro Asn Leu His Gly Pro Glu Ile Leu Asp Val Pro Ser Thr Val Gln Lys Thr 1890
 Phe Val Thr His Pro Gly Tyr Asp Thr Gly Asn Gly Ile Gln Leu Pro Gly Thr Ser Gly 1910
 Gln Gln Pro Ser Val Gly Gln Gln Met Ile Phe Glu Glu His Gly Phe Arg Arg Thr Thr 1930
 Pro Pro Thr Thr Ala Thr Pro Ile Arg His Arg Pro Arg Pro Tyr Pro Pro Asn Val Ala 1950
 Leu Ser Gln Thr Thr Ile Ser Trp Ala Pro Phe Gln Aso Thr Ser Glu Tyr Ile Ile Ser 1970
 Cys His Pro Val Gly Thr Asp Glu Glu Pro Leu Gln Phe Arg Val Pro Gly Thr Ser Thr 1990
 Ser Ala Thr Leu Thr Gly Leu Thr Arg Gly Ala Thr Tyr Asn Ile Ile Val Glu Ala Leu 2010
 Lys Asp Gln Gln Arg His Lys Val Arg Glu Val Val Thr Val Gly Asn Ser Val Asn 2030
 Glu Gly Leu Asn Gln Pro Thr Asp Asp Ser Cys Phe Asp Pro Tyr Thr Val Ser His Tyr 2050
 Ala Val Gly Asp Glu Trp Glu Arg Met Ser Glu Ser Gly Phe Lys Leu Leu Cys Gln Cys 2070
 Leu Ser Phe Gly Ser Gly His Phe Arg Cys Asp Ser Ser Arg Trp Cys His Asp Asn Gly 2090

Fig. 5E

Val Asn Tyr Lys Ile Gly Glu Lys Trp Asp Arg Gln Gly Glu Asn Gly Gln Met Met Ser²¹²⁰
 Cys Thr Cys Leu Gly Asn Gly Lys Gly Glu Phe Lys Cys Asp Pro His Glu Ala Thr Cys²¹⁴⁰
 Tyr Asp Asp Gly Lys Thr Tyr His Val Gly Glu Gln Trp Gln Lys Glu Tyr Leu Gly Ala²¹⁶⁰
 Ile Cys Ser Cys Thr Cys Phe Gly Gly Gln Arg Gly Trp Arg Cys Asp Asn Cys Arg Arg²¹⁸⁰
 Pro Gly Gly Glu Pro Ser Pro Glu Gly Thr Thr Gly Gln Ser Tyr Asn Gln Tyr Ser Gln²²⁰⁰
 Arg Tyr His Gln Arg Thr Asn Thr Asn Val Asn Cys Pro Ile Glu Cys Phe Met Pro Leu²²²⁰
 Asp Val Gln Ala Asp Arg Glu Asp Ser Arg Glu²²³⁰

Fig. 5F

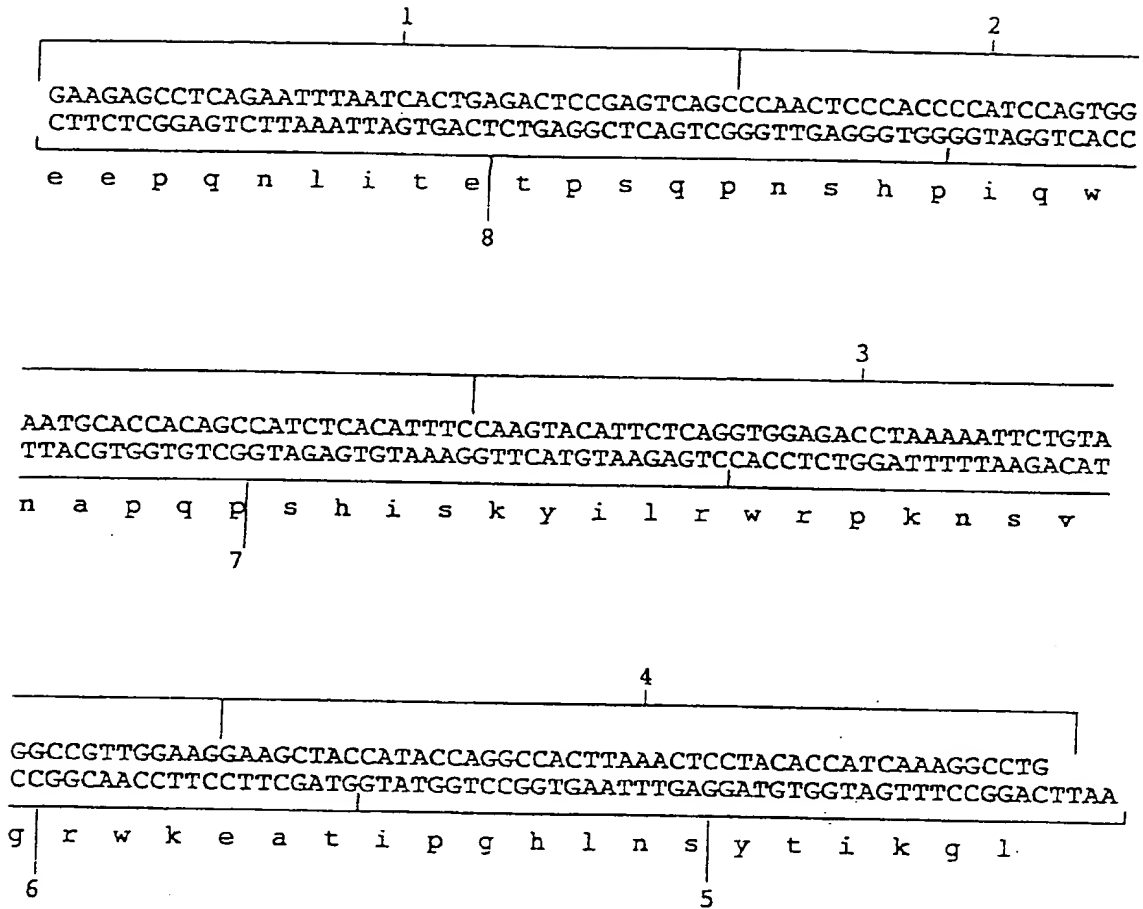


Figure 6 Linker 5 showing the eight constituent oligonucleotides

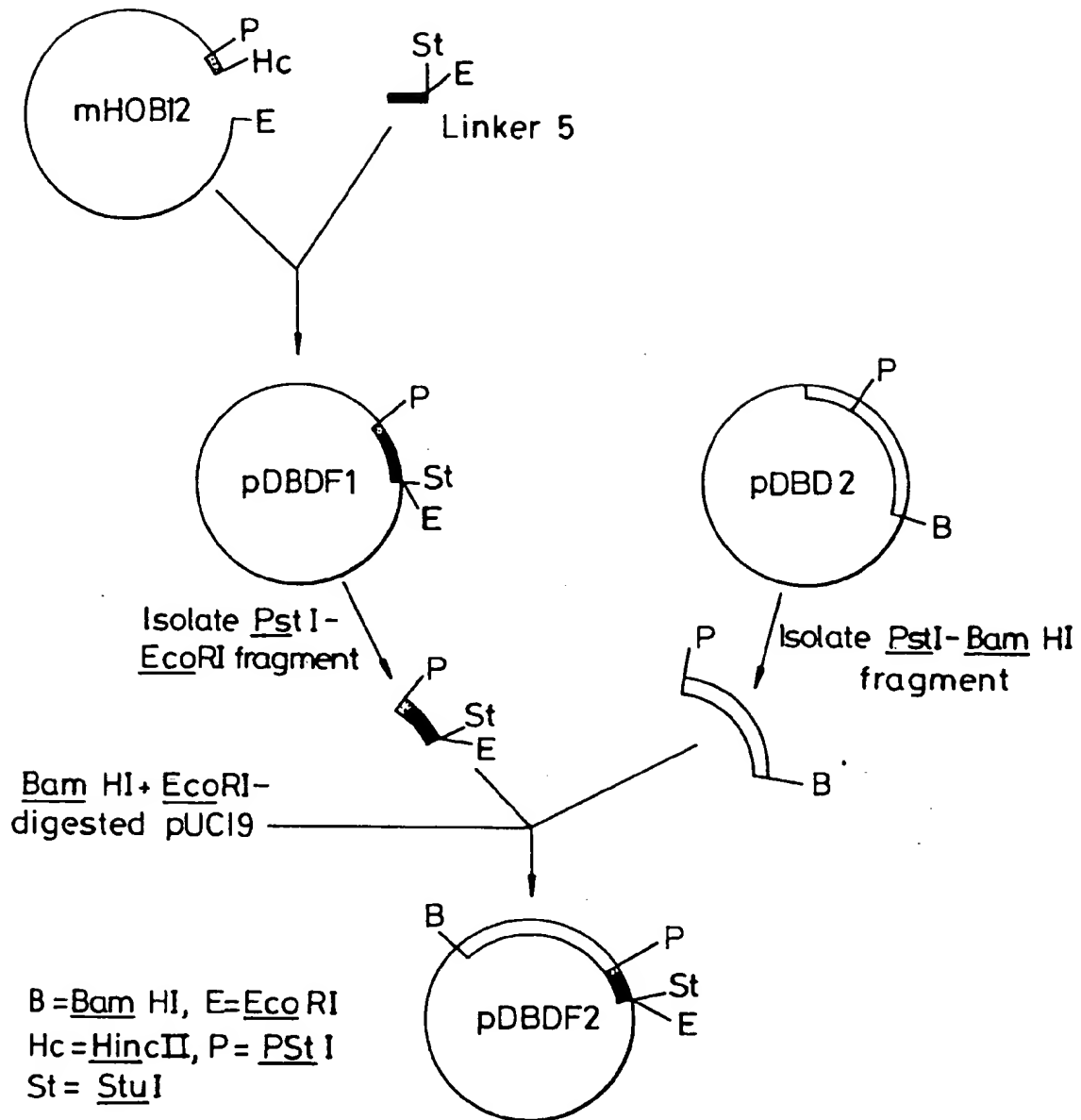


Fig. 7 Construction of pDBDF2

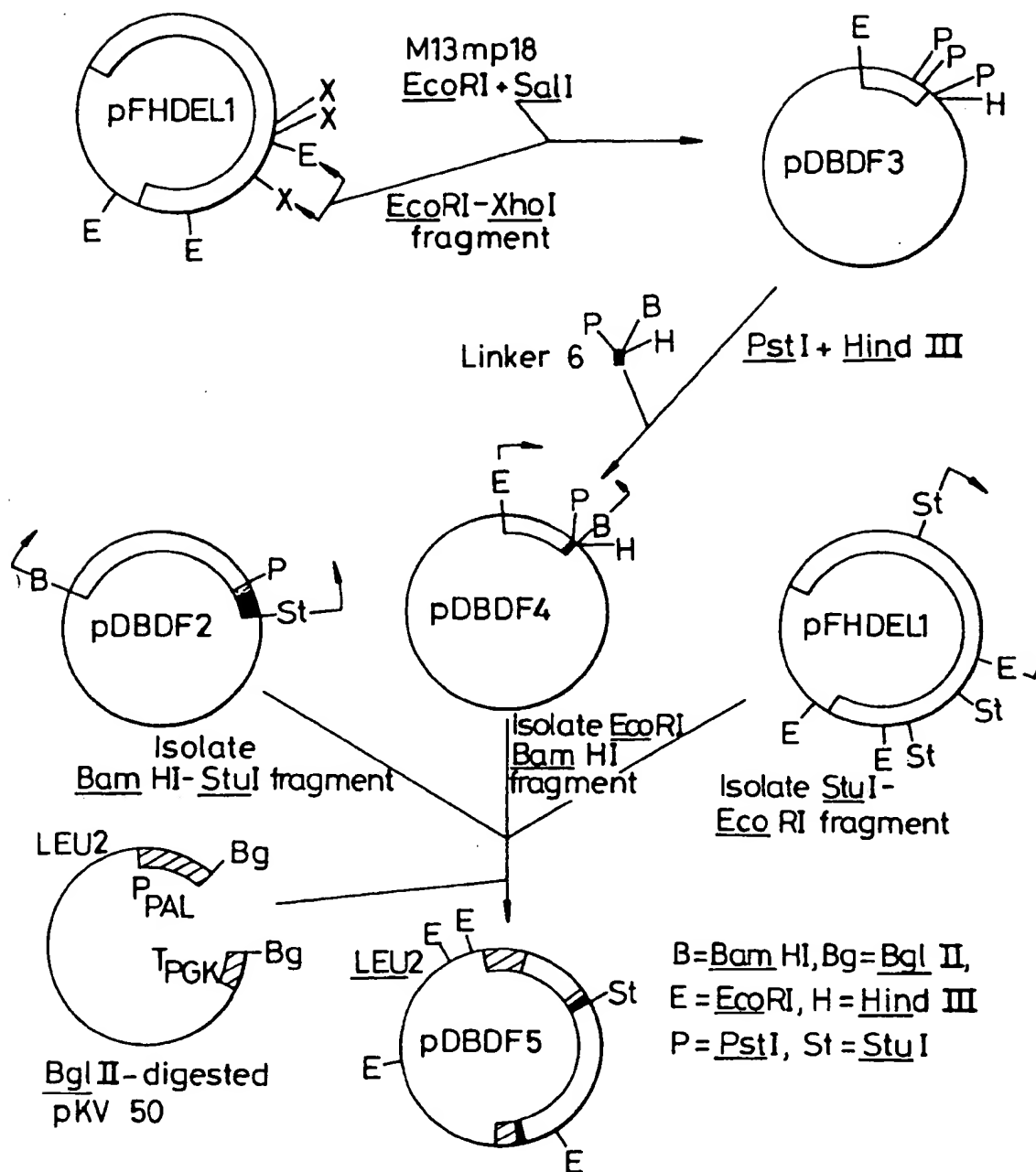


Fig. 8 Construction of pDBDF5

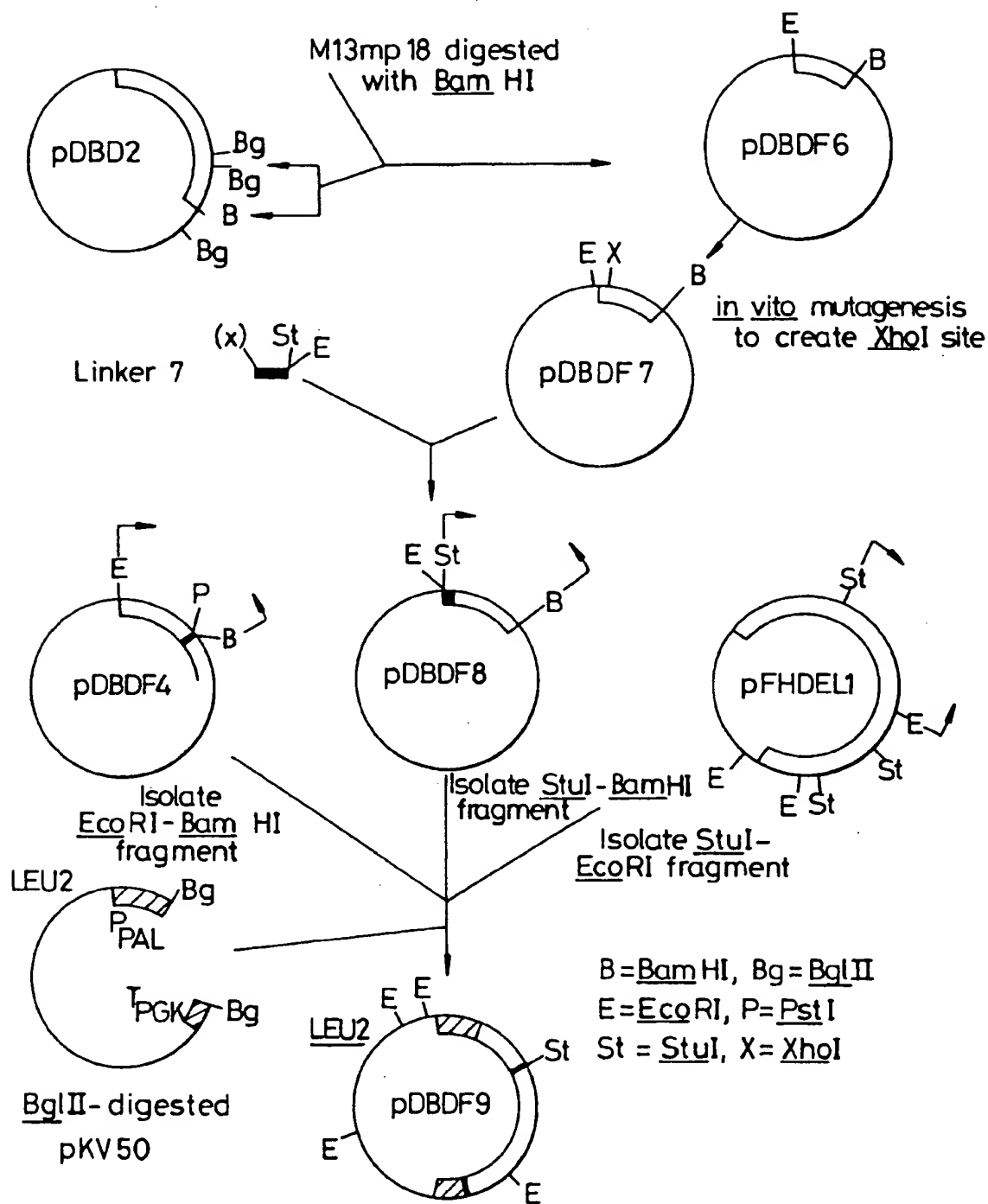


Fig. 9 Construction of pDBDF9

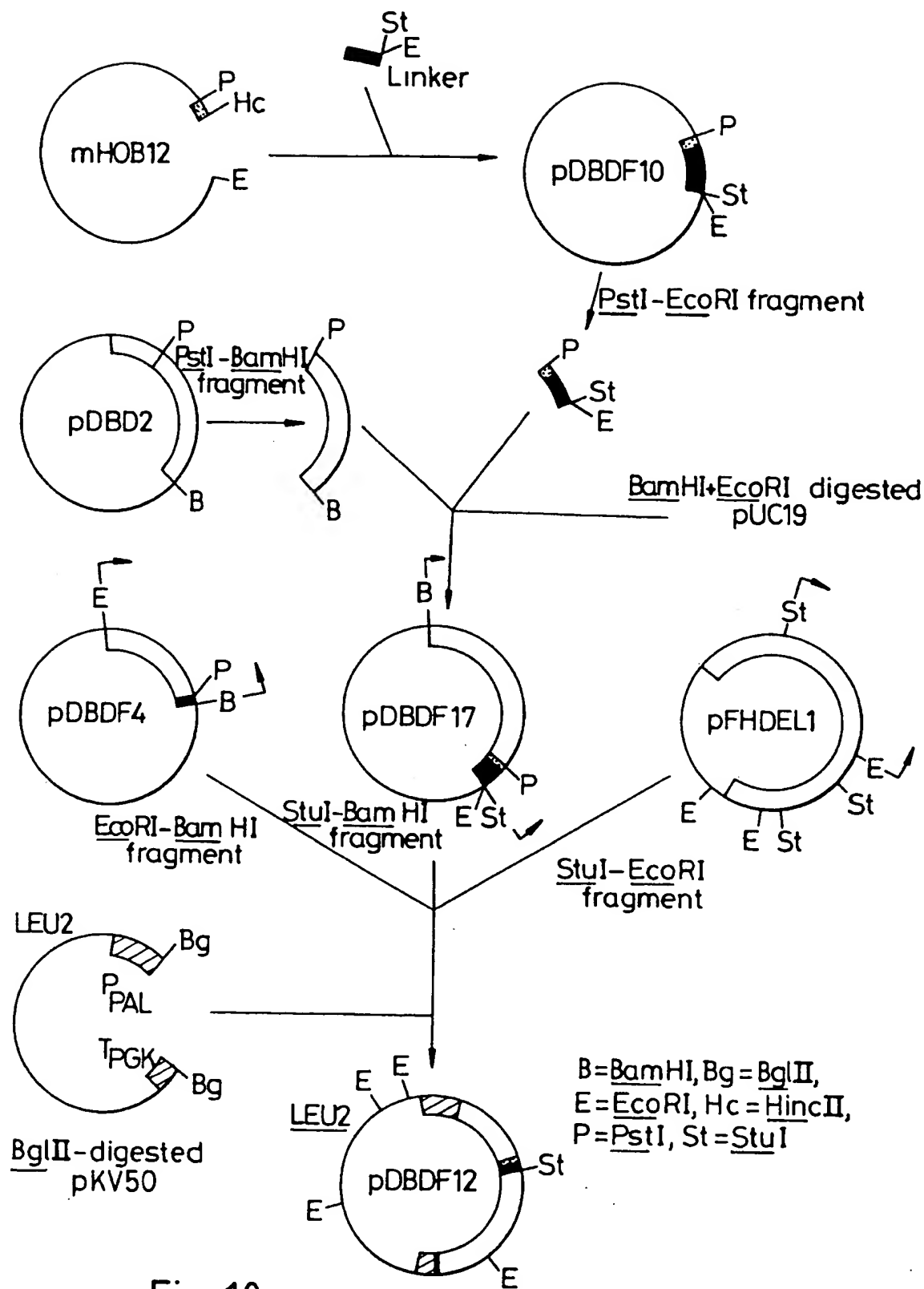


Fig. 10 Construction of pDBDF12

Figure 11

Name: pFHDEL1
 Vector: pUC18 Amp^r 2860bp
 Insert: hFNCdNA - 7630bp

